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# Thiosemicarbazone derivatives, thiazolyl hydrazones, effectively inhibit leukemic tumor cell growth: Down-regulation of ribonucleotide reductase activity and synergism with arabinofuranosylcytosine

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ABSTRACT

Cellular growth inhibition exerted by thiosemicarbazones is mainly attributed to down-regulation of ribonucleotide reductase (RNR) activity, with RNR being responsible for the rate-limiting step of *de novo* DNA synthesis. In this study, we investigated the antineoplastic effects of three newly synthesized thiosemicarbazone derivatives, thiazolyl hydrazones, in human HL-60 promyelocytic leukemia cells.

The cytotoxicity of compounds alone and in combination with arabinofuranosylcytosine (AraC) was determined by growth inhibition assays. Effects on deoxyribonucleoside triphosphate (dNTP) concentrations were quantified by HPLC, and the incorporation of radio-labeled $^{14}$C-cytidine into nascent DNA was measured using a beta counter. Cell cycle distribution was analyzed by FACS, and protein levels of RNR subunits and checkpoint kinases were evaluated by Western blotting.

VG12, VG19, and VG22 dose-dependently decreased intracellular dNTP concentrations, impaired cell cycle progression and, consequently, inhibited the growth of HL-60 cells. VG19 also lowered the protein levels of RNR subunits R1 and R2 and significantly diminished the incorporation of radio-labeled $^{14}$C-cytidine, being equivalent to an inhibition of DNA synthesis. Combination of thiazolyl hydrazones with AraC synergistically potentiated the antiproliferative effects seen with each drug alone and might therefore improve conventional chemotherapeutic regimens for the treatment of human malignancies such as acute promyelocytic or chronic myelogenous leukemia.



## 1. Introduction

Thiosemicarbazones have been thoroughly investigated over decades, raising great interest to pharmaceutical and clinical research owing to their antibacterial, antiviral, antifungal, and antineoplastic effects (Beraldo and Gambino, 2004). To date, it has been proven that the antiproliferative effects of thiosemicarbazone derivatives are linked to the inhibition of ribonucleotide reductase (RNR) (Kalinowski et al., 2009), an enzyme being essential for *de novo* DNA synthesis by converting ribonucleoside diphosphates into deoxyribonucleoside diphosphates (Jansson et al., 2015). RNR protein levels are highly expressed in tumor cells rendering the iron-dependent enzyme an excellent target for cancer chemotherapy (Ahmad et al., 2015).

Mammalian RNRs consist of two large α- and two small β-subunits forming an active α2β2 holoenzyme tetramer (Aye et al., 2015). The large homodimeric R1 effector binding subunit (α2)

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harbors a substrate and two different allosteric sites, while the small homodimeric R2 subunit (β2) comprises two diferric iron centers each stabilizing a tyrosyl radical. Completing RNR biosynthesis, the R1 subunit is encoded by gene *Rrm1*, whereas *Rrm2* and *p53R2* encode the homologue R2 isoforms. p53R2 protein expression is induced by DNA damage and contributes dNTPs for DNA repair in the $G_0/G_1$ cell cycle phase (Bourdon et al., 2007). The R2 subunit serves as a target for anticancer drugs inhibiting the nonheme iron subunit by either metal ion chelation or radical scavenging of the tyrosyl radical (Shao et al., 2013), a mechanism being also attributed to thiosemicarbazones (Kalinowski et al., 2009).

Hydroxyurea (HU) was the first RNR inhibitor being introduced into clinical practice (Singh and Xu, 2016) and has been used for decades in the treatment of chronic myeloid leukemia (CML), acute myeloid leukemia (AML), and other neoplastic malignances (Saban and Bujak, 2009; Sterkers et al., 1998; Tennant, 2001). Triapine (3AP) is a thiosemicarbazone derivative and inhibits the R2 subunit through chelation of the iron centers (Aye et al., 2015). 3AP, clinically tested for CML and other solid tumors, acts as RNR inhibitor in HU-resistant tumors (Finch et al., 2000), particularly by building up metal-bound 3AP complexes, which are able to generate reactive oxygen species (ROS) (Popovic-Bijelic et al., 2011) and, subsequently, inhibit R2 (Zhu et al., 2009). However, several Phase I and II clinical trials reported on toxic side effects of 3AP (Attia et al., 2008; Murren et al., 2003) leading to the development of di(2-pyridyl) ketone thiosemicarbazones (DpT), 2-benzoylpyridine thiosemicarbazones (BpT), and 2-acetylpyridine thiosemicarbazones (ApT), all of which demonstrated a more pronounced antiproliferative effect (Lovejoy et al., 2012) but less toxicity (Yuan et al., 2004) in various human tumor cell lines.

Acute promyelocytic leukemia (APL) is an infrequent subtype of AML with typical biological traits, often associated with leucopenia and severe coagulopathy, resulting in hemorrhagic complications and/or thrombosis (Lo-Coco et al., 2016). Combination therapy of arabinofuranosylcytosine (AraC) with an anthracycline has been the only choice for APL treatment until the late 1980s, being accountable for decreasing relapses in APL with high WBC counts (more than $10 \times 10^9$/L) (Kelaidi et al., 2009; Sanz et al., 2009). Meanwhile, therapeutic strategies have been innovated remarkably, and substantial improvement in the outcome of patients has taken place since the implementation of *all-trans*-retinoic acid (ATRA) especially in combination with arsenic trioxide (ATO) (Mi et al., 2012). Despite amelioration of chemotherapy was gained by the application of high-dose AraC to high-risk patients leading to even further improvement of APL survival (Murren et al., 2003), several groups suggested that avoiding AraC in the chemotherapy of APL might reduce AraC-induced toxicity without increasing relapses (Estey et al., 1997; Sanz et al., 2004). However, Sanz and coworkers found that addition of AraC to combined idarubicin and ATRA treatment in consolidation for high-risk patients led to a substantially higher antileukemic activity which was paralleled by an increased but tolerable toxicity (Sanz et al., 2010). Although the role of AraC in APL has remained somewhat controversial, the majority of studies suggest a reduction of relapse risk through addition of AraC, and that combining AraC with ATRA might have a supra-additive (synergistic) effect (Sanz and Lo-Coco, 2011). Taken together, AraC is considered to be the second most effective drug in AML/APL, surpassed only by ATRA. Accordingly, the omission of AraC in APL patients led to an increased risk of relapse (Ades et al., 2006), indicating enough evidence to support the administration of AraC in combined chemotherapeutic regimens for APL, thereby allowing lower cumulative anthracycline doses, the latter being responsible for long-term cardiotoxicity (Creutzig et al., 2010).

Aim of the present study was to gain a deeper insight into the anticancer efficacy of 40 newly synthesized thiosemicarbazone derivatives, thiazolyl hydrazones, using qualitative and quantitative structure-activity relationship (QSAR) techniques (Siddique et al., 2017). This modern drug design theory relates structural and chemical properties of a compound to its biological activities. Three compounds showed pronounced growth inhibition of human HL-60 acute promyelocytic leukemia cells and were therefore elected for further investigations including the identification of potential synergistic effects.

The most apparent advantage of combining drugs is the achievement of additive and/or synergistic properties through alteration of specific molecular pathways resulting in a decrease of drug resistance, drug dosage and, accordingly, drug toxicity. AraC is well-known especially for affecting intracellular dCTP pools (Gandhi et al., 1997; Seymour et al., 1996; Wills et al., 2000) thus causing synergism with various inhibitors of RNR (Fritzer-Szekeres et al., 2008; Horvath et al., 2005, 2006; Saiko et al., 2011, 2007, 2015). The different findings described in the clinical studies led us to the assumption that the combination of AraC with putative inhibitors of RNR could enhance its antineoplastic behavior, thereby reducing its toxicity thus preserving the beneficial effects seen with this treatment option.

Following this strategy, we tested the effects of binary mixtures of the most promising thiazolyl hydrazones (VG12, VG19, VG22) with AraC to investigate potential additive and/or synergistic effects in the human HL-60 cell line. Additionally, we examined the consequences of drug treatment on RNR *in situ* activity by measuring the incorporation of radio-labeled $^{14}$C-cytidine into nascent HL-60 tumor cell DNA. Alterations of deoxyribonucleoside triphosphates (dNTPs), the products of RNR metabolism, were analyzed using a specific HPLC method developed by our group. Finally, protein levels of RNR subunits (R1, R2, and p53R2) were determined by Western blotting and upon treatment with compounds, cell cycle perturbations were measured by FACS analysis.

## 2. Materials and methods

### 2.1. Chemicals and supplies

Thiazolyl hydrazones were synthesized and provided by the Department of Pharmaceutical Sciences, Birla Institute of Technology, Mesra, India (Siddique et al., 2017). Structural formulas, molecular weight, and biological activity are given in Fig. 1. AraC and all other chemicals and reagents were commercially available and of highest purity.

### 2.2. Cell culture

The human HL-60 promyelocytic leukemia cell line was purchased from ATCC (American Type Culture Collection, Manassas, VA, USA). Cells were grown in RPMI 1640 medium with L-glutamine and 25 mM HEPES supplemented with 10% heat inactivated fetal calf serum (FCS), 1% L-glutamine and 1% penicillin-streptomycin at 37 °C in a humidified atmosphere containing 5% $CO_2$ using a Heraeus cytoperm 2 incubator (Heraeus, Vienna, Austria). Cell counts were determined using a microcellcounter CC-110 (SYSMEX, Kobe, Japan). Cells growing in the logarithmic phase of growth were used for all experiments described below.

### 2.3. Growth inhibition assay

HL-60 cells ($0.1 \times 10^6$ per ml) were seeded in 25 cm$^2$ Nunc tissue culture flasks and incubated with increasing concentrations of drugs at 37 °C under cell culture conditions. Stock solutions were diluted in DMSO. Cell counts were determined after 24, 48, and 72 h